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Modifications in the biochemical properties of *Helianthus annus* due to infection by *Pseudomonas syringae*, *Alternaria* spp and *Cercospora* spp

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Abstract: *Three organisms namely Pseudomonas syringae ,Cercospora spp and Alternaria spp were isolated from infected spots on a sunflower leaf. The development of yellow fluorescence on Kings B agar media and production of toxin proved the presence of Pseudomonas syringae. A number of significant changes in the plant properties were observed after 15days of infection by the isolated pathogens. An assay of the enzyme activity of phenylalanine ammonia lyase (PAL) at 270nm and polyphenol oxidase at 280 nm indicated an increase in the concentration of trans-cinnamic acid and o-quinones respectively after 5 days of infection and a decrease in their concentration after 15 days of infection. However, the β carotene content of the sunflower petals remained almost unaffected due to the infections. The antifungal activity of Pseudomonas syringae against Cercospora spp and Alternaria spp was indicated during co infection on sunflower through the modification of viscosity of the sunflower oil as well as reduction of germ tube length during co infection with the fungal pathogens. The use of lipid metabolism blockers showed that there was a decrease in the germ tube length of the spores of the isolated pathogens. Further, by slide bio assay, it was found that fungicides like Bavistin and Blitox can be used effectively to control infection of Alternaria spp and Cercospora spp. Also the use of bactericides like Ambistryn can also be used in controlling infection by Pseudomonas syringae on Helianthus annus.*

Keywords: *Pseudomonas, Ambistryn, Bavistin, Blitox, o-quinones,trans-cinnamic acid.*

I. Introduction:

Helianthus annus, belonging to the Asteraceae family, is rich in vitamin A and E, omega 3 fatty acids, magnesium, iron, copper, zinc and tannins. The uses of the sunflower plant are limitless. It ranks as one of the four major crops of global importance and has an annual production of approximately 26 million tonnes [1]. Owing to its antioxidant and anti-inflammatory properties, it has widespread medicinal uses as well. The use of the sunflower oil as a source of biodiesel as well as for human consumption has further increased its production in the recent years [2].

The utilities of the sunflower plant get limited due to several abiotic and biotic factors. Factors like temperature, oxygen availability and moisture play important roles in regulating the quality of sunflower seeds [2]. The sunflower plant is also susceptible to infection by bacteria, fungi, nematodes and visuses [3]. The intensity of damage to sunflower plants and its oil production capacity is greatly affected by the time of infection. It has been observed that infections in mature plants do not always lead to losses in yield as compared to infections in the seed[4]. About 90 diseases of the sunflower plant have been identified till now [5] and it has been noted that fungal diseases cause serious damages to the plant [3].

Fungal infections like the leaf blight disease caused by *Alternaria* spp [6] as well as the leaf spot disease of *Cercospora* spp can cause a lot of damage to the sunflower plants. *Alternaria* spp was first identified as a pathogen causing diseases in the sunflower plant in 1978 [7]. Symptoms appear as circular, dark brown to black lesions with concentric rings ranging from 0.2 mm to 0.5 mm in diameter. Lesions eventually enlarge in size and coalesce and may be accompanied with organelle destruction by toxic compounds released by the fungi [8], causing blighting of leaves. In case of infections caused by *Cercopsora* spp there is lesion formation which initially occurs on older leaves. Lesions at maturity are 1/8 inch in diameter[3] and appear light gray-colored to dark tan with a brown to purple border [9].A diagnostic feature is the presence of tiny black dots (pseudostromata) in leaf substomatal cavities inside the greyish-tan lesions.

In case of bacterial infections like infection by *Pseudomonas syringae*,initial symptoms of bacterial leaf spot are small, water-soaked spots that are visible from both sides of the leaf. The lesions usually are limited

by leaf veins and thus have an angular, square, or rectangular appearance. These water-soaked lesions rapidly turn brown and with aging may dry out and become papery. Also apical chlorosis may occur in the sunflower plant due to infection by *Pseudomonas syringae* [10].

Infection by such phyllosphere pathogens can greatly alter the various biochemical characteristics of the sunflower plant and thus limit the various uses of the sunflower plant. In this paper, we have tried to study the various biochemical modifications namely modifications in the enzyme activity, viscosity of sunflower oil and pigment production occurring in the sunflower plants due to infection by *Alternaria* spp, *Cercospora* spp and *Pseudomonas syringae* as well as find probable ways to eliminate infection by these pathogens. A thorough study of the modifications caused in the plant due to infection is carried out, and a proper understanding of the decreased yield of sunflower oil is obtained through the set of experiments conducted.

II. Materials And Methods:

2.1 Isolation of the pathogens:

Infected sample of sunflower plant was taken and the infected area (nature and % of infection) and the infected leaf dimensions, fresh and dry weight was physically characterized. Slants were prepared with two media-- nutrient agar (NA) media for isolating bacterial pathogen and potato dextrose agar (PDA) media for isolating fungal pathogen. Several small sections 5 to 10 mm square from the margin of the infected lesion to contain both diseased and healthy–looking tissue were excised from the leaf. These were placed in a surface disinfectant solution (0.1% mercuric chloride) for 1 min. This may be diluted, in which case the material must be immersed for a longer time, making sure that the surface is immersed properly in the disinfectant solution. It was then washed three times in sterilized distilled water in watch glass with forceps, and was inoculated on the slants.The inoculated slants were then kept at incubation- NA slants for 24hrs at 28^oC and PDA slants for 72hrs at room temperature.

2.2 Detection of toxin production by the infection causing pathogens:

Day 1: A loopful of bacterial sample was inoculated into a fresh nutrient broth tube from a pure culture of *Pseudomonas syringae*(obtained after growth on N.A slants from the excised leaf sections followed by growth on Kings B agar media) under the laminar air flow. It was then incubated for 24 hours in 37^o C in an incubator shaker.

Day 2: Overnight grown culture of *Pseudomonas syringae* was centrifuged at about 5000 r.p.m for 15mins and the supernatant was collected.100 microliter of supernatant was injected at the base of a leaf of a healthy sunflower plant grown under controlled conditions and observed for the formation of lesions.

2.3Modifications in the biochemical characteristics of the sunflower plant due to infection by the isolated pathogens:

2.3.1. Modification in the activity of phenylalanine ammonia lyase (PAL) due to infection by the isolated pathogens:

2gms of infected (infection period of 5 days) and uninfected sunflower leaves were taken and macerated with silica and extracted with Tris-HCl buffer (pH=8.5). The extract was mixed with 1ml of phenylalanine solution. After mixing and incubating for 5 minutes, 1 ml of water was added into each tube and the O.D of trans-cinnamate was measured at $O.D_{270\ nm}$ by spectrophotometer. The same experiment was repeated with infected leaves where the infection was allowed to span for a period of 15 days.

2.3.2. Modification in the polyphenol oxidase (PPO) activity due to infection by the isolated pathogens:

2gms of infected (infection period of 5 days) and uninfected sunflower leaves are taken and macerated with silica and extracted with Tris-HCl buffer (pH=8.5). It was mixed with 1ml of tyrosine solution. After mixing and incubating for 5 minutes 1 ml of water was added into each tubes and the O.D of trans-cinnamate was measured at $O.D_{280\ nm}$ by spectrophotometer. The same experiment was repeated with samples where the infection was allowed to proceed for a time span of 15 days.

2.3.3. Modification in the viscosity of the sunflower oil due to infection by the isolated pathogens:

1 gm of petals were weighed on a balance both from non-infected and infected sunflower and macerated in a motor pestle with the help of silica powder. Then 35 ml of hexane solution was added to each macerated sample and then transferred to a conical. Then the entire solution was filtered and poured into Oswald viscometer. In both case, the filtered solution was added at one opening and suction was applied so that the level of liquid changes. The suction is removed after which the liquid falls to its original position A owing to the force of gravity. The time required for the meniscus to move from points A to B was noted with the help of a stopwatch.

2.3.4. Modification in the β-carotene pigment content due to infection by the isolated pathogens:

Sample preparation:1ml of the spore suspension of *Alternaria* spp, *Cercospora* spp and 1ml of culture of *Pseudomonas syringae* was injected near the base of the fresh sunflower petals of three sunflower plants. After 5 days of infection, the petals were plucked and 5gms of it was weighed. 5gm of petals from a fresh uninfected sunflower was also taken. The infected and uninfected samples were taken in mortar pestles and they were crushed with silica. The samples were macerated till they took a paste like appearance. In to a conical flask containing 50ml of 95% ethanol,5 g of the macerated sample was placed and maintained at a temperature of 70-80^{o}C in a water bath for 20minutes with periodic shaking. The supernatant was decanted, allowed to cool and its volume was measured by means of a measuring cylinder and recorded as initial volume. The ethanol concentration of the mixture was brought to 85% by adding 15ml of distilled water and it was further cooled in a container of ice water for about 5minutes. The mixture was transferred in to a separating funnel and 25ml of chloroform was added and the cooled ethanol was poured over it. The funnel was swirled gently to obtain a homogenous mixture and it was later allowed to stand until two separate layers were obtained.The bottom layer was run off into a beaker while the top layer was collected in to a 250ml conical flask. The bottom layer was transferred in to the funnel and re-extracted with 10ml chloroform for 5-6 times until the extract became fairly yellow. The entire chloroform was collected in to 250ml conical flask and transferred in to separating funnel for re-extraction with 50ml of 80% ethanol. The final extract was measured and poured in to sample bottles for further analysis[11]

2.3.5. To study the kind of interaction between the isolated pathogens:

Grease free glass slides were taken. 20µl of spore suspension of *Cercospora* spp and *Alternaria* spp was added in two separate slides. 10µl of culture suspension of *Pseudomonas syringae* was added in each of the slides except the control where 10µl of sterile water was added along with the spore suspensions.The slides were then covered with cover slips and kept in petri-dishes with moistened filter papers in it. Then the plates were kept in a tray with moistened absorbent cotton inside it and covered with a glass plate cover. The observations were taken after 24 hours of incubation.

2.3.6. Control of infection of the isolated pathogens:

Grease free glass slides were taken. 20µl of spore suspension of *Cercospora* spp was taken in four slides and *Alternaria* spp was added in four separate slides. A pinch of Bavistin, Blitox and a lipid metabolism blocker was added in the slides except the control where 10µl of sterile water was added along with the spore suspensions.The slides were then covered with cover slips and kept in petri-dishes with moistened filter papers in it. Then the plates were kept in a tray with moistened absorbent cotton inside it and covered with a glass plate cover. The observations were taken after 24 hours of incubation.

III. Results:

3.1. Isolation of the pathogens:

Slant	Growth Characteristics
2 Nutrient agar slants	Bacterial growth was observed. Yellow colored colonies were seen around the inoculated sample.
2 Potato Dextrose agar slants	Fungal growth was observed. Cottony overgrowth was seen along with blackish spores.

The bacteria was characterised by gram staining, its dimensions were measured by micrometry, motility tested by hanging drop experiment, pigment production verified by growing on Kings B media and finally identified by 16sr RNA analysis to be *Pseudomonas syringae*. Further, the fungal pathogens were identified by their spore characters revealed by fungal staining to be *Alternaria* spp and *Cercospora* spp.

3.2. Detection of toxin production by the infection causing pathogens

A pale yellow spot was observed in the leaf in the area where bacterial supernatant was added. This signified that there was toxin production by *Pseudomonas syringe* and was subsequently collected in the supernatant fraction. This toxin may be implicated later with the leaf spot development and the exact HPLC characterisation of the toxin fraction may be done to for a more precise analysis on the toxin-host interaction. The development of this toxin indicated that one of the modes of infection used by the bacteria is by the production of toxin.

3.3. Modifications in the biochemical characteristics of the sunflower plant due to infection by the isolated pathogens

3.3.1. Modification in the phenylalanine ammonia lyase(PAL) activity due to infection by the isolated pathogens:

After 5 days of infection, there was an increase in the concentration of trans-cinnamic acid as well as an increase in the activity of the enzyme phenylalanine ammonia lyase as compared to the uninfected sample (refer to TABLE 1). However, when the same experiment was allowed to span for a period of 15 days, there was a marked reduction in the concentration of trans-cinnamic acid as well as in the activity of the enzyme phenylalanine ammonia lyase (refer so TABLE 2).

Table 1: After 5 days of infection:

Sample infected with	Absorbance at 270 nm after 5 days of incubation	Concentration µg/ml X 10^{-2}	Enzyme activity (µg/ml) min^{-1} x 10^{-2}
Uninfected	0.215	1.23	0.246
Cercospora spp.	0.232	1.33	0.266
*Alternar*ia spp.	0.226	1.29	0.258
Pseudomonas syringae	0.237	1.36	0.272

Table 2: After 15 days of infection:

Sample infected with	Absorbance at 20 nm (after 15 days of infection)	Concentration of trans cinnamic acid (µg/ml) x 10^{-2}	Enzyme activity of PAL(µg/ml) min^{-1} x 10^{-2}
Uninfected sample	0.202	1.16	0.232
Cercosporaspp	0.157	0.9022	0.180
Alternariaspp	0.168	0.9655	0.193
Pseudomonas syringae	0.171	0.9827	0.196

3.3.2. Modification in the polyphenol oxidase of the sunflower leaves due to infection by the isolated pathogens:

The concentration of o-quinones as well as the activity of the enzyme PPO underwent a reduction after 15 days of infection as compared to samples where the infection was allowed to proceed for 5 days of infection (refer to TABLE 3 and TABLE 4).

Table 3: After 5 days of infection:

Sample infected with	Absorbance at 280 nm (After 5 days on infection)	Concentration of quinones (mg/ml)	Enzyme activity of PPO (mg/ml) min^{-1}
Uninfected sample	0.478	478	95.6
Cercospora spp	0.565	565	113
Alternaria spp	0.549	549	109.8
Pseudomonas syringae	0.527	527	105.4

Table 4: After 15days infection**:**

Sample infected with	Absorbance at 280 nm (After 15 days on infection)	Concentration of quinones (mg/ml)	Enzyme activity of PPO (mg/ml) min^{-1}
Uninfected sample	0.482	482	96.4
Cercospora spp	0.410	410	82
Alternaria spp	0.423	423	84.6
Pseudomonas syringae	0.445	445	89

3.3.3. Modifications in the viscosity of the sunflower oil due to infection by the isolated pathogens:

The viscosity of the sunflower oil seemed to be reduced significantly as compared to oil from uninfected sunflower plants with the maximum reduction in case of infection by *Cercospora* spp and minimum in case of infection by *Psedomonas syringae*.(refer to TABLE 5) Also it was seen that when *Cercospora* spp and *Pseudomonas syringae* were made to co infect the plant, the viscosity, though less that of the uninfected sunflower plant, was more than that when *Cercospora* spp was allowed to infect the plant alone. Again, when *Alternaria* spp and *Pseudomonas syringae* were made to co-infect the plant, the viscosity was more than that when *Alternaria* spp was allowed to infect the plant alone (refer to TABLE 6)

Table 5

Infection by	Experiment No.	Time(sec)	Average time 't' sec	Viscosity
Non infected	A	65		
	B	65	**65**	0.124
	C	66		
***Cercospora* spp**	A	47	**47**	
	B	47		0.090
	C	48		
***Alternaria* spp**	A	48	**49**	
	B	49		0.093
	C	49		
Pseudomonas syringae	A	56	**56**	
	B	56		0.107
	C	55		

Table 6

Infection by	Experiment No.	Time(sec)	Average time 't' sec	Viscosity
Cercospora* spp and *Pseudomonas syringae	A	52	**52**	0.100
	B	52		
	C	53		
Alternaria* spp and *Pseudomonas syringae	A	54	**54**	0.103
	B	54		
	C	54		
***Cercospora* spp and *Alternaria* spp**	A	46	**45**	0.086

3.3.4. Modification in the β-carotene pigment due to infection by the isolated pathogens:

The β carotene pigment content of the sunflower petals undergoes very minor changes due to infection by the isolated pathogens. This may be due to sample to sample variations in the sunflowers used as indicated by TABLE 7.

Table 7

Absorbance at	Sample infected with	Optical density after 10 days of infection	Concentration of β carotene(µg/ml)
436nm	Uninfected sample	0.424	0.0339
436nm	*Cercospora* spp	0.423	0.0338
436nm	*Alternaria* spp	0.422	0.0337
436nm	*Pseudomonas syringae*	0.422	0.0337

3.3.5. To study the interaction between the isolated pathogens:

Co-infection of *Pseudomonas syringae* with *Cercospora* spp. or *Alternaria* spp. caused a reduction of germ tube lengths of both the fungal pathogens as indicated by TABLE 8.

Table 8

Organism	No of divisions covered by the ocular micrometer(Control)	No of divisions covered by the ocular micrometer(with Pseudomonas syringae)	Final Reading(Controlµm)	Final Reading(With *Pseudomonas syringae*,µm)
Alternaria spp	4 4	2 3	15.4	9.63
*Cercospo*ra spp	5 4	3 3	17.33	11.55

3.3.6. Control of infection of the isolated fungal pathogens:

The use of lipid metabolism blockers as well as fungicides like Blitox (copper fungicide) and Bavistin (carbendazim) indicated antifungal activities against *Cercospora* spp and *Alternaria* spp due to reduction in

their germ tube lengths (refer to TABLE 9). Also the growth of *Pseudomonas syringae* was seen to be significantly reduced due to addition of standard bactericides like Ambistryn (refer to TABLE 10).

Table 9

Organism	Final Reading(µm) Control	Final Reading(µm) With Lipid metabolism blocker	Final Reading(µm) With Bavistin fungicide	Final Reading(µm) With Blitox fungicide
Alternaria spp	15.4	7.78	3.85	5.78
Cercospora spp	17.33	9.625	5.78	7.78

Table 10

Organism	Optical density after 24 hrs of inoculation (600nm)	Optical density 24 hrs after addition of Ambistryn
Pseudomonas syringae	0.1820	0.0113

IV. Discussions:

- Phenylalanine ammonia lyase (PAL) is the first committed enzyme that is involved in the phenyl propanoid biosynthetic pathway [12].It is involved in the production of polyphenolic compounds like flavonoids, lignin and phenyl propanoids [13]in plants and its activity is very sensitive to the damage to plant tissues caused due to infection by invading pathogens or other means. The reduction in the activity of the enzyme after 15 days of infection indicates the reduced ability of the plant to protect itself from infection by production of such polyphenolic compounds. This indicates that PAL is involved in providing short term resistance to the plant and in case of infections that proceed for a longer time span, the activity of PAL reduces since the overall damage to the plant has an effect on the basal activity levels of many cellular proteins and enzymes.
- The weakening of the defense mechanism of the plant due to prolonged time of infection or pathogen mediated destruction of defense system of the plant may also be responsible for the reduction in the PPO activity after 15 days infection .
- The reduction in the viscosity of the sunflower oil in infected samples maybe be due to the fact that the infecting pathogens are involved in utilization of some of the monounsaturated and polyunsaturated fatty acids, which are the main components of the sunflower oil, as a source of their nourishment hence leading to the reduction in the viscosity of the sunflower oil. This reduction may also be a reflection of the reduced ability of the plant to produce all the components required for production of sunflower oil of standard viscosity owing to weakening of the plant due to infection by the pathogens.However, it is highly unlikely that the pathogens would be able to utilise them in the form in which it had been produced in the plant. So there is always a possibility that the lipid metabolism is active in these organisms.
- During co-infection of the fungal and bacterial pathogens there was an increase in viscosity as compared to infection by only the fungal pathogen. This indicated a kind of antagonistic relationship between the bacterial and the fungal pathogens due to which during co infection, the infectivity of one of the pathogens is being suppressed by the other.
- The minor reduction in the β carotene content as compared to uninfected samples may be due to sample variations in the sunflowers used or due to some damages in the enzyme and pigment systems in the sunflower plant because of infection by the isolated pathogens. Both the cases indicate that the infection route used by *Alternaria* spp, *Cercospora* spp and *Pseudomonas syringae* does not affect the β carotene content of the sunflower petals to a great extent.
- On measuring the germ tube lengths of *Cercospora* spp and *Alternaria* spp in presence of water as well as after addition of *Pseudomonas syringae* culture, it was observed that there was a decrease on the germ tube lengths of the fungal pathogens in the presence of the bacteria. This indicated that *Pseudomonas syringae* may have some anti-fungal activity against *Cercospora* spp and *Alternaria* spp during co infection. However, the exact nature of the antifungal activity is yet to be elucidated.
- The reduction in the germ tube lengths of the two fungal pathogens on addition of lipid metabolism blockers further helped to support the hypothesis that the lipid metabolism is highly active in the pathogenic isolates. Also it was seen that the addition of two standard fungicides Bavistin and Blitox can be used effectively to reduce the growth of the two fungal pathogens *Cercospora* spp and *Alternaria* spp. Again, *Pseudomonas syringae* infection in sunflower plants could be effectively controlled by addition of standard anti-bacterial compounds like Ambistryn. In this way large scale wilting and destruction of sunflower crops can be controlled.

V. Conclusion:

Many biochemical properties of the sunflower plant get distinctly modified due to infection by *Cercospora* spp, *Alternaria* spp and *Pseudomonas syringae*. The activities of the enzymes PAL and PPO show a

marked increase during the early stages of infection indicating an activation of the defence mechanism of the plant. However the activities of the same enzymes reduce when infection spans for around 15 days presumably due to weakening of the defence system. Also the viscosity of the sunflower oil obtained from the petals undergoes a reduction due to infection. Thus infections by such pathogens can lead to a wide range of changes in the biochemical properties of the sunflower plants and needs to be controlled by relatively cheap antifungals like Blitox and Bavistin and antibacterial compounds such as Ambistryn. So if at the outset of the infection, such compounds can be sprayed at definite concentrations, such infections can be controlled leading to increased sunflower oil and pigment production.

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